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GENETIC TRANSFORMATION OF ANTISENSE ACC OXIDASE IN *Carica papaya L.* CV. SEKAKI VIA PARTICLE BOMBARDMENT

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ABSTRACT

Papaya (*Carica papaya L.*) is a very important crop in many tropical countries. Climacteric fruits such as papaya are usually harvested once they have reached maturity, which then undergoes rapid ripening during transit and storage. Hence, papaya is highly susceptible to physiological disorders, mechanical damage and fruit over ripening that can cause postharvest losses of papaya production. Therefore, this study was conducted on genetic transformation of antisense ACC oxidase and its effects on papaya fruit ripening. Through anti-sense technology, down regulation of the ACC oxidase gene results in the suppression of ethylene production, thereby delaying fruit ripening that can produce papaya with better quality. Embryogenic callus of Sekaki papaya were bombarded with the pCAMBIA 1301 that contains the antisense ACC oxidase gene driven and flanked by constitutively CaMV35S promoter and NOS terminator also hygromycin (*hpt*) resistance gene as selectable marker. Helium gas pressure (1350 psi), 1.0 µm gold particle size and two types of parameters manipulated in the bombardment condition were, the number of bombardment (single or double) and the target position (3 cm, 6 cm and 9 cm). Results showed that double bombardment per sample and 6 cm of target position gave the highest percentage of callus survival which is 88% after 4 weeks in a selection medium containing 50mg/l hygromycin. The PCR analysis for antisense ACC oxidase, *hpt* and *Nad5* genes done on the extracted callus genome gave positive results. The remaining bombarded explants are being maintained to obtain viable regenerates for further analyses.

ABSTRAK

Betik (*Carica papaya L.*) adalah salah satu tanaman yang penting di kebanyakan negara tropika. Buah klimakterik seperti betik pada kebiasaannya akan dipetik apabila mencapai tahap kematangan, yang mana ia akan melalui proses pemasakan buah dengan cepat semasa penghantaran dan penyimpanan. Oleh yang demikian, betik mempunyai pendedahan yang sangat tinggi terhadap masalah fisiologi, kerosakan luaran dan buah yang terlebih masak yang mana akan menyebabkan kerugian pada penghasilan betik selepas tuai. Oleh itu, kajian transformasi genetik terhadap gen antisense ACC oxidase telah dijalankan di mana ianya memberi kesan terhadap pemasakan buah betik. Melalui teknologi antisense yang dijalankan, penghasilan yang rendah pada gen ACC oxidase akan merencatkan penghasilan etilena yang mana dapat melambatkan proses pemasakan buah seterusnya menghasilkan buah betik dengan kualiti yang baik. Kalus embriogenik betik sekaki yang digunakan telah ditransformasi dengan pCAMBIA 1301 yang mengandungi gen antisense ACC oxidase diapit dengan pencetus konstitutiv CaMV35S dan terminator NOS. Plasmid yang digunakan juga mempunyai gen kerintangan terhadap higromisin (*hpt*) sebagai penanda pemilihan. Penggunaan tekanan gas helium (1350psi), saiz partikel emas (1.0 µm) dan pemilihan dua parameter yang telah dimanipulasi iaitu bilangan bedilan (1 atau 2 kali) dan juga jarak di antara mikroprojektil dengan tisu sasaran (3,6,9 cm) telah dilakukan sewaktu proses pembedilan. Keputusan yang diperolehi menunjukkan bahawa pembedilan sebanyak 2 kali per sampel dan jarak pembedilan 6cm memberikan peratusan kebolehidupan kalus yang paling tinggi iaitu 88% selepas 4 minggu pengkulturan di dalam media pemilihan higromisin (50mg/L). Analisis PCR yang dijalankan terhadap gen antisense ACO, gen *hpt* dan gen *Nad5* hasil daripada ekstraksi kalus menunjukkan keputusan yang positif. Kalus yang masih tinggal disimpan dengan baik untuk regenerasi dan analisis yang seterusnya.

Key words: *Carica papaya L.*, particle bombardment, antisense ACC oxidase, embryogenic callus

INTRODUCTION

Papaya is a tropical and subtropical crop which is considered to be one of the most important sources of vitamins A and C (Bhattacharya & Khuspe, 2001) also become one of the major economic crop in many tropical countries. *Carica papaya L. cv.* Sekaki also known as 'Hong Kong' is the second most popularly cultivated variety in Malaysia after Eksotika. It is a cross-pollinated variety and a

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prolific bearer (60-70 tonnes/ha/year) with medium sized fruit of 1.5 – 2 kg. Sekaki fruit is attractive with smooth, even-coloured and freckle-free skin. In climacteric fruits, a dramatic increase in ethylene biosynthesis promotes the subsequent steps of fruit ripening, involving biochemical and physiological changes (Pereira *et al*., 2005). However, papaya fruit is susceptible to overripening caused by ethylene and the technology was used today to extend the shelf life of papaya is based on the control of ethylene action and production (Lo´pez-Go´mez *et al*., 2009). Papaya's overripening gave the problems for market industry because of the papaya shipments arriving at terminal markets have a range of disorders associated with over-ripeness, mechanical injury and parasitic diseases (Capellini *et al*., 1988). For the other reason, fruits transported for long periods under refrigeration to prevent damage and delay their ripening also have the tendency reduce their quality. Therefore, studies on ethylene production in papaya fruit have focused on measurements of ACC oxidase activity (Lo´pez-Go´mez *et al*., 2009) which done by antisense inhibition of ACC oxidase that has been possible to delay papaya fruit ripening for the better purpose. They are many conventional method of papaya cultivars can be done for controlling the fruit ripening process which one of the technique by spraying the fruits with ethylene gas. However, it met very limited success because of the other constraint. Thus, the rapid development in biotechnologies, especially those involved in the genetic transformation of plants, has made it possible to introduce selected genes into plants for controlling the over ripening fruits. *Agrobacterium* and biolistic are the most commonly employed gene delivery systems but physical nature of particle bombardment can potentially overcome many of the biological barriers, such as host-range specificity associated with *Agrobacterium* (Fisk & Dandekar, 1993). Fitch *et al*. (1990) was the first to successfully transform *via* particle bombardment and regenerate transgenic papaya plants derived from papaya zygotic embryos, hypocotyl sections, or somatic embryos of 'Sunrise Solo' and 'Kapoho Solo'. This work was undertaken with the aim to generate transgenic papaya plants containing a fragment of the ACC oxidase gene in the antisense orientation to block ethylene production and delay the ripening rate. The objective of this study is to establish an optimum and stable transformation system using particle bombardment that allows the introduction of selected genes into Sekaki papaya and enables regeneration into plants.

MATERIALS AND METHODS

Plant material and callus induction

Immature fruit of *Carica papaya L. cv.* Sekaki was collected from papaya orchard at Broga, Semenyih, Selangor. Immature green papaya fruit (90- to 120-day-old) from the mid position of fruit bunch was selected as a plant material. Seeds were then surface sterilized using ethanol (70%) for 1min followed by treating with Clorox (30%) containing 2 drops of Tween 20 for 10 min under sterile condition. Immature zygotic embryos were inoculated on petri dishes containing two types of basal medium MS (Murashige & Skoog 1962) supplemented with 30g/l of sucrose, 400 mg/l glutamine, 50 mg/l myo inositol, 4g/l gelrite and 10 mg/l 2, 4-D. The pH was adjusted to pH 5.8. The culture was incubated at 25°C ± 2°C in the dark. After 5-6 weeks of culture, embryogenic callus was selected as a target explant for bombardment.

DNA/Microprojectile Preparation and bombardment conditions

The selected embryogenic callus that use for bombardment was subcultured to the fresh callus induction medium supplemented with the 9g/l of Mannitol and 9g/l Sorbitol, providing high osmoticum conditions to make the explants suitable for shooting. Embryogenic callus (100-150) were placed in the centre of 9 cm diameter Petri dishes. After incubation for ±16 hours, the embryogenic callus were immediately subjected to microprojectile bombardment with the Biolistic Particle Delivery PDS-1000/He system (Bio-Rad). The plasmid used in this work, pCAMBIA 1301 plasmid construction that contains the antisense ACC oxidase gene (ACO), flanked and driven by the constitutively CaMV 35S promoter and NOS terminator. This plasmid construct also contains the hygromycin (*hpt*) resistance gene as the selectable marker. The mixture of 1.0 µM (Bio-Rad) gold microcarriers were coated with the plasmid DNA. Gold particles of 6 mg were sterilized in 100 µL absolute ethanol (2 min). After washing with sterile water twice, particles were resuspended in 100 µL sterile distilled water. The mixture for coating was prepared as follows: 100 µL of particle suspension were mixed with 5 µL x (1µg/µL) of plasmid DNA, 50 µL $CaCl_2$ (2.5 M) and 20 µL spermidine (0.1 M). The particle/DNA suspension was vortexed and left (10 min) at room temperature. The DNA-coated particles were pellet by centrifugation at 10,000 rpm (10 sec). The supernatant was completely removed and discarded. The pellet was resuspended in 60 µL absolute ethanol. Particle/DNA mixture (8 µL) was placed in the centre of microcarrier. There are various parameter employed for optimizing conditions of bombardment of embryogenic callus of Sekaki papaya to get the highest rate of transformation frequency. The selected parameters for manipulation were target distance which means distance between microcarrier and target (3, 6, and 9 cm) and number

of shots per plate (once and twice time). Another bombardment conditions such as rupture disc pressure was carried out at (1350 psi) and vacuum pressure (27 mmHg) were maintain constant. Petri dishes containing unbombarded tissues were taken as control. After bombardment, the cultures were maintained on the same medium in dark at 25°C ± 2°C. Bombarded calli were then transferred to callus induction medium without adding manitol and sorbitol after 1 weeks of cultured for callus proliferation.

Selection of bombarded cells

After two weeks, bombarded calli were transferred to callus induction medium supplemented with 50mg/L of hygromycin B followed by subcultured every 14 days (for at least teo subcultures) on the same fresh medium for propagating of the transformed calli. The cultures were maintained on the same medium in dark at 25°C ± 2°C. Callus that survived and proliferated were calculated and recorded for determination the best bombardment condition of papaya.

Analysis of the transformed cells by Polymerase Chain Reaction (PCR)

Genomic DNA of transformed and untransformed callus was isolated according to the CTAB extraction method (Doyle & Doyle modification 1990). The presence of antisense ACO, *hpt* and *Nad5* genes in transformants was confirmed by PCR amplification using specific primers. The sequences of antisense ACO primers used for amplification of 667bp fragment were 5'-CCC GGT NAC CTT AAG ATG GAG AAC TTC C-3' forward primer and 5'-CCC AGA TCT CCA CAA TAG AGT GGC GC-3' reverse primer. The PCR cycle for antisense ACO gene were: 95ºC (3 minit), 95ºC (30 sec) 63ºC (30 sec), 72ºC (1 min) (35 cycles) and further extension 72ºC (5 min). Primer sequence for the *hpt* gene were F: 5' ACA GCG TCT CCG ACC TGA TGC 3' and R: 5' AGT CAA TGA CCG TGT TAT GCG 3' with the expected 600bp gene amplification. The PCR cycle for *hpt* gene were: 94ºC (5 min), 94ºC (30 sec), 60ºC (1 min), 72ºC (2 min) (30 cycles) 72ºC (7 min). For *Nad5* gene, the primers used here F:5' TAG CCC GAC CGT AGT GAT GTT AA 3' and R: 5'ATC ACC GAA CCT GCA CTC AGG AA 3'. The expected size was 800 bp in length. The PCR cycle for *Nad5* gene were: 95ºC (1 min), 96ºC (30 sec), 58ºC (30 sec), 72ºC (1 min) (29 cycles) and 72ºC (3 min). Concentration of PCR reagent for all the genes are as followed: 1.25 U *Taq* DNA Polymerase, 2 mM dNTP, 3 mM $MgCl_2$, 5X reaction buffer, 20 µmol primer and 1 µl (template). The total volume (25 µl) end up with additional of distilled water. PCR products were separated by 1% agarose gel electrophoresis using TAE buffer and visualised with ethidium bromide.

RESULTS

Callus proliferation

The initiation of callus from immature embryo was observed after two weeks of cultivation onto callus induction medium under dark condition. Another 6 weeks of culture, embryogenic callus were produce in a form of several creamy white to light yellow like embryos. Most of the proliferated callus are friable and suitable for transformation and potentially selected for the target explants (Fig1a).

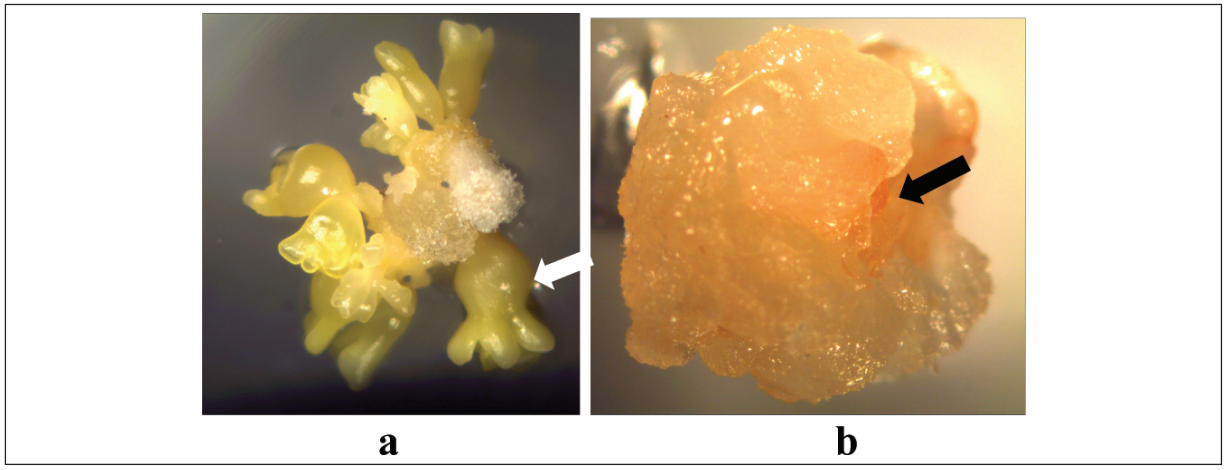

Fig 1. a) Embryogenic callus of Sekaki papaya on MS medium + 10 mg/l 2,4-D.
b) Callus induction in selection medium after 4 weeks of bombardment under bombardment condition (1350 psi, double shots and 6 cm target distance). Each arrow with different colour represented; white arrow -Embryogenic callus; black arrow-callus induction

Bombardment condition and selection of putative transgenic callus

For this purpose, the plasmid pCAMBIA 1301 harboring the economically important gene antisense ACO, which confers the transgenic plants delaying the fruit ripening and the selectable marker gene *hpt* was used in bombardment experiments. After two weeks of bombardment, less of callus produce was obtained at the selection medium (hyg 50 mg/l) and some of the callus become dark brownish and not survive. However, a large mass of proliferated callus from the origin of bombarded callus was clearly observed another two weeks of culture (Fig1b). Data of the percentage callus survival were recorded for each treatments. Results showed that, manipulation of biolistic parameters influencing gene delivery in papaya. Target distance also influenced the succes in DNA delivery into embryogenic callus. It was found that shooting of callus at target distance (6 cm) gave the highest percentage of callus survival compared to the other target distance. There were suggested that 6 cm gave the optimum distance for shooting the target cells. From the result, shorter target distance (3 cm) promoted the low percentage of survival calli. These might happened because shooting of callus at shorter flight distance led to hardly damaged most of the cells and the effects on the cells growth and proliferation. Meanwhile, callus that shooting at the longer target distance (9 cm) gave the low percentage of callus survival than 6 cm because a little amount microprojectile can reach the target cells and lowering the transformation rate of the callus. Result reported here also showed all of the callus transformed at target distance (9 cm) with the single shot per plate were not survive. Data summarized in Table 1 indicated that target distance (6 cm) with double shoots per plate together with applied pressure of 1350 psi gave the highest percentage of callus survival (88%) on selective medium. From all of this observation that have beeen done, it can be suggested that flight distance of 6 cm, double shots per plate and applied pressure of 1350 psi are the optimal physical conditions which gave the highest frequency of putative transgenic callus based on survival onto selective media. Half of the survival callus in a plate have been selected for PCR analysis. The remaining callus were maintained in a good condition for shoots and plantlet regeneration.

Table 1. Percentage of callus survival after 4 weeks of culture in selection medium (hyg 50 g/L) under different biolistic conditions

Target distance	No. of bombardment Callus survival (%)	
	Single	**Double**
3cm	75% (a)	20% (c)
6cm	79% (b)	88% (d)
9cm	-	65% (e)

(*) letter that show in bracket represent the treatment of each bombardment condition

PCR analysis

Molecular conformation was performed on the DNA level using PCR amplification. Specific primers flanking antisense ACO, *hpt* and *Nad5* gene used for PCR amplification. Figure 2A and 2B showed antisense ACO and *hpt* putative positive transgenic clones having the expected amplification products. Positive control for antisense ACO and *hpt* gene showed 667bp and 600bp bands respectively. The expected size of *Nad5* gene which was housekeeping gene for plant genome was 800bp that obtained either from transformed callus or non transformed callus. However, no bands appear at Figure 2A (lane 3), Figure 2B and Figure 2C (lane 2) which gave negative result of PCR analysis for the respective genes. This could be happened because of low concentration of the samples were taken for extraction and samples that obtained were not enough for requirements of the PCR analysis. Related from that, specific primers that have been used for respective genes could not detect and amplified the selected sequence from the DNA samples due to low concentration subsequently were affected the PCR analysis. Therefore, samples with no band appear for antisense ACO and *hpt* gene analysis may concluded that genes were not integrated to this samples and consequently become non-transform callus. Although the percentage of survival callus with target distance (6 cm) and single shot per plate which is treatment (a) promoted high percentage however figure 2A, 2B and 2C for these treatment showed negative result of PCR analysis for antisense ACO, *hpt* and *Nad5* genes. These could be suggested that not all the survival callus were transformed with antisense ACO and *hpt* genes which means may be the callus resistance in the selection medium.

DISCUSSION

This study describes an efficient biolistic-mediated transformation system for economically important papaya plants using embryogenic callus derived from immature embryo explants. The used of embryogenic callus for target cells in this present study due to the potential of the cell to develop into plants. Mousavi *et al*., (2009) also reported embryogenic calli showed the highest average number of blue spots per shot compared to leaf while

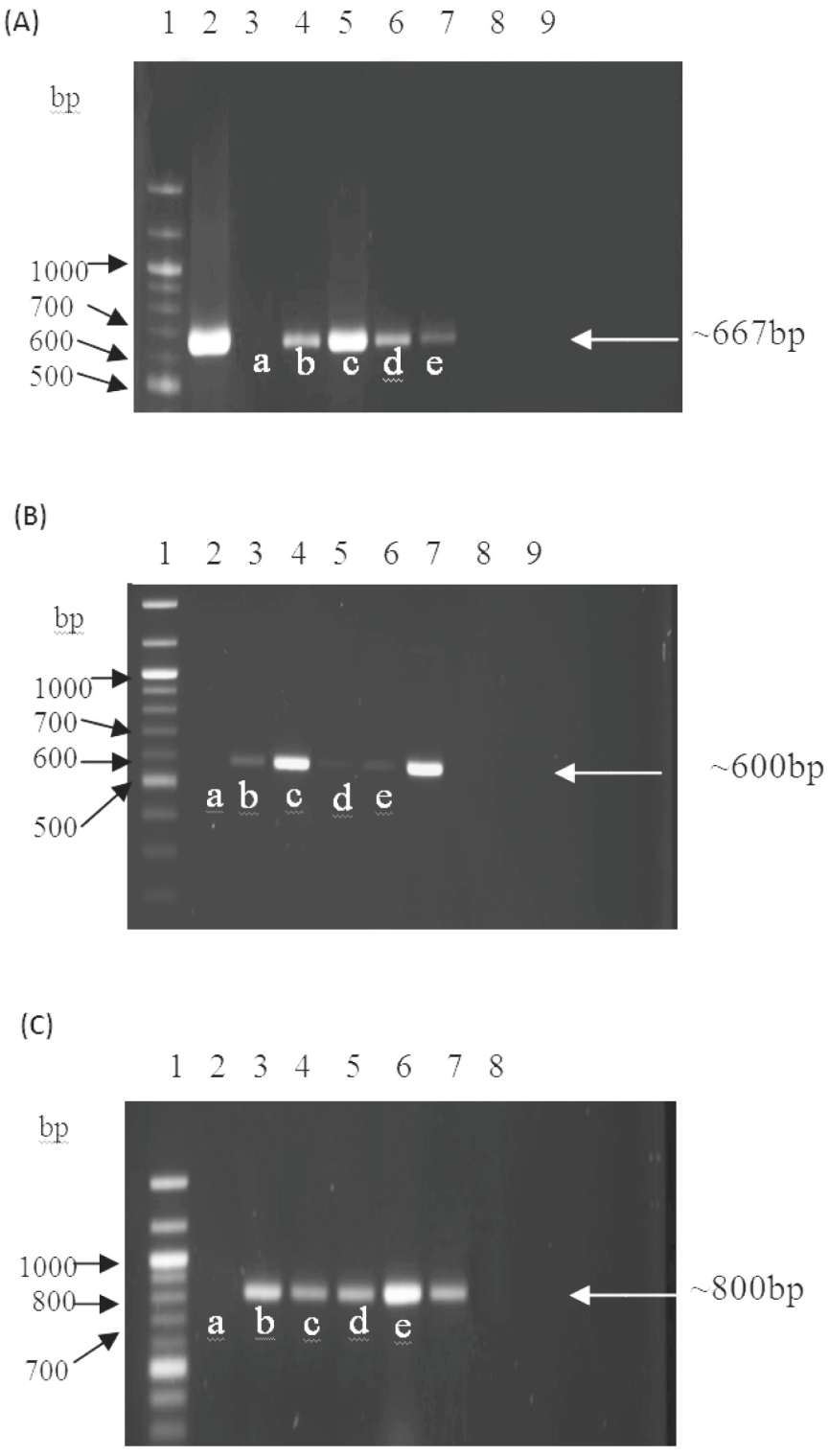

Fig 2. **(A)** PCR analysis of extracted callus for antisense ACO gene. Lane 1: 100bp marker. Lane 2: positive control of pCAMBIA 1301. Lane 3-7: DNA from transformed callus. Lanes 8: DNA from non-transformed callus. Lanes 9: negative control (without template) .Letter that show under the band represent the treatment used; **(B)** PCR analysis of extracted callus for *hpt* gene. Lane 1: 100bp marker. Lane 7: positive control pCAMBIA 1301.Lane 2-6: DNA from transformed callus. Lanes 8: DNA from non-transformed callus. Lanes 9: negative control (without template). Letter that show under the band represent the treatment used; **(C)** PCR analysis of extracted callus for *Nad5* gene. Lane 1: 100bp marker. Lane 2-6: DNA from transformed callus. Lanes 7: DNA from non-transformed callus. Lanes 8: negative control (without template). Letter that show under the band represent the treatment used.

no spots appeared after bombardment of the roots. The developed protocol involved prior optimization of embryogenic callus induction system using immature embryos. The optimal bombardment conditions was obtained from this study which gave the highest frequency of transformation. This suggested the microprojectile bombardment method was applicable to transform wide range of crops (Christou, 1997).

An important aspect in transformation *via* biolistics is the damage to the target tissue during microparticle penetration into the cell. To minimize this problem, the target cells are usually plasmolysed by an osmotic treatment (Brettschneider *et al.,* 1997). The elevation of osmotic pressure is generally obtained by the addition of mannitol, sorbitol, maltose or sucrose to the culture medium (Brettschneider *et al.,* 1997). According to Wang *et al.* (2003), post bombardment osmotic treatments favor a higher recovery of stable events. The same effect was demonstrated in this present study by using high concentration of mannitol (9 g/L) and sorbitol (9 g/L) for more than 16 hours incubation before and after bombardment promoted optimum osmoticum condition to protect the cells. Increased osmoticum concentrations may enable to protect the cells from leakage and bursting, and may also improve particle penetration itself (Hagio, 1998). Armaleo *et al*. (1990) obtained higher transformation rates when yeast cells were subcultured for some hours before and after particle bombardment on medium with a high osmolarity. Vain *et al.,* (1993) also reported that there was a gain in transient expression when embryos were maintained for more 16 hours after bombardment in high osmotic concentration medium. The beneficial effect of this treatment is believed to come from the reduced turgor pressure so that more cells can be penetrated by particles without osmotic disruption (Brettschneider *et al*., 1997).

Acceleration pressure is another parameter that influences the efficiency of biolistic to deliver DNA into various explants. The ability of the microcarrier particles to successfully penetrate different cell types is highly dependent on the helium gas pressure (Kikkert, 1993). In this present work, only one applied pressure (1350 psi) which include at higher range of pressure was used for bombardment. In stable transformation experiments Brettschneider *et al*. (1997) found significant higher transformation rates at 1300 psi than at to 900 psi. This could indicate that cells which are competent for embryogenesis are located in deeper cell layers and that these are penetrated at a higher helium pressure. Lower expression at reduced pressures could be correlated to the poor penetration capability of the microcarriers as they approach the recipient tissues (Mousavi *et al*., 2009)

The distance from the microprojectile launch site to the biological target can affect the velocity of microprojectiles and consequently transformation rates (Petrillo *et al*., 2008). For this purpose of study, higher percentage of transformation rate was detected when explants were placed at 6 cm of flight distance. This present study was supported by Tadesse *et al.* (2003) who had reported that a higher number of transient GUS expression as well as stable transformation events at a microcarrier flying distance of 6 cm than at 12 cm. This present result was closely related with Petrillo *et al*. (2008) which found a higher number of expression units were obtained when samples were located 6.6 cm away from the launch platform, followed by 9.9 and 13.2 cm.

Apart from, to increase the possibility of hitting a greater number of cells capable to undergo somatic embryogenesis, the influence of more than one shot per plate was tested. Two shots per plate produce 88% survival rate of callus transform in this study may be because of the plasmid concentration that penetrates to the targeted cells more higher compared to one shot per plate. Petrillo *et al*., (2008) observed more than one consecutive shot on the same plate resulted in increased GUS transient expression. However, there were no transgenic plants produced when three shots were used. Three shots per plate probably caused more injury to the explants, which impaired cell proliferation and regeneration. Reggiardo *et al.* (1991) found that more than one bombardment per plate had deleterious effects on barley and maize cells.

CONCLUSION

This study conclusively demonstrated that genetic transformation of papaya can be done by particle bombardment by manipulating the bombardment conditions. Embryogenic callus were selected as a targeted cells because of the cells potential to regenerate into plants successfully. Therefore, the protocol utilized may be useful to introduce agronomical important genes into papaya plants.

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